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Review

Proteasome inhibitors: from research tools to drug candidates

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Abstract

The 26S proteasome is a 2.4 MDa multifunctional ATP-dependent proteolytic complex, which degrades the majority of cellular polypeptides by an unusual enzyme mechanism. Several groups of proteasome inhibitors have been developed and are now widely used as research tools to study the role of the ubiquitin–proteasome pathway in various cellular processes, and two inhibitors are now in clinical trials for treatment of multiple cancers and stroke.

Keywords: Proteasome inhibitor; Ubiquitin–proteasome pathway; Drug candidate

1. Introduction

The ubiquitin–proteasome pathway is the major proteolytic system in the cytosol and nucleus of all eukaryotic cells. This ATP-dependent pathway was discovered more than 20 years ago [1,2], but the involvement of the proteasome particle was demonstrated only in the late 1980s [3]. Much of our initial understanding of the importance of this pathway in the regulation of different cellular processes came from biochemical studies in extracts of mammalian cells and genetic studies in yeast (reviewed in [4]). However, knowledge about its physiological roles in mammalian cells was slow to develop until cell-permeable proteasome inhibitors were developed, which greatly simplified such studies. Introduction of proteasome inhibitors led to the demonstration that the proteasome catalyzes the degradation not only of the majority of short-lived but also of long-lived proteins, which comprise the bulk of proteins in mammalian cells [5,6]. Studies using these compounds have demonstrated that the ubiquitin–proteasome pathway is responsible for the breakdown of a large variety of cell proteins and is essential for many cellular regulatory mechanisms (Table 1). For example, cell cycle progression is controlled by the proteasomal degradation of cyclins and inhibitors of cyclin-dependent kinases [7], while degradation of transcriptional regulators, such as c-Jun, E2F-1 and β-catenin (see [8] for review) is essential for the regulation of cell growth and gene expression. Similarly, degradation by the proteasome of activated protein kinases, e.g. src and protein kinase C [9,10], is critical for the termination of certain signal transduction cascades.

The ubiquitin–proteasome pathway also plays an important role in the regulation of many physiological processes as well as in the development of a number of major human diseases. For example, degradation of the tumor suppressor p53 (reviewed in [8]), and $p27^{Kip1}$ inhibitor of cyclin-dependent kinases [11] can promote tumorigenesis. As discussed below, proteasomal degradation of the IκB inhibitor of the transcription factor NF-κB is essential for the development of inflammatory response [12,13]. Furthermore, the ubiquitin–proteasome pathway also plays an essential role in immune surveillance [14], muscle atrophy [15], regulation of metabolic pathways [16–18], acquisition of long-term memory [19], as well as in regulation of circadian rhythms [20] and photomorphogenesis in plants [21].

Another important function of the ubiquitin–proteasome pathway is in the selective removal of mutant, damaged and misfolded proteins. It has been claimed recently that a large fraction (perhaps even one-third) of newly synthesized proteins cannot fold properly and are degraded by the proteasome [22]. In addition, post-synthetic damage, such as damage by oxygen radicals or isomerization of aspartic acid residues, which occurs for example

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Table 1
Physiological functions and selected substrates of ubiquitin–proteasome pathway

Function	Substrate
Cell cycle progression	$p27^{Kipl}$, p21, cyclins
Oncogenesis	p53, $p27^{Kipl}$, bax, IκB
Apoptosis	Bcl-2, cIAP, XIAP
Regulation of gene expression	c-Jun, E2F1, IκB, β-catenin
Inflammation	IκB, p105 precursor of NF-κB
Long-term memory	Protein kinase A (regulatory subunit)
Regulation of metabolic pathways	Ornithine decarboxylase, HMG-CoA reductase
Immune surveillance	Most cytosolic and nuclear proteins
Protein quality control	CFTRΔF508, α_1-antitrypsin (Z-variant), aged calmodulin
Regulation of circadian clock	Timeless
Photomorphogenesis in plants	Hy5

during in vitro 'aging' of calmodulin [23], can trigger rapid degradation of a protein. Moreover, abnormal proteins, which cannot fold because of mutation and are degraded by proteasome (Table 1), are important causes of various genetic diseases, including cystic fibrosis [24,25] and hereditary α_1-antitrypsin deficiency, a cause of emphysema [26].

The majority of substrates of this pathway are marked for degradation by covalent attachment of multiple molecules of ubiquitin, a small 8 kDa protein (Fig. 1). First, the ubiquitin is attached to a free amino group on the substrate, generally through an isopeptide bond to the amino group of a lysine side chain [8]. A chain of ubiquitins is formed as several ubiquitin molecules are processively added to the Lys-48 of the preceding ubiquitin. This task is accomplished by three enzymes, E1, E2 and E3. E1 activates the ubiquitin molecule in an ATP-dependent process and transfers it to one of at least 15 different E2 ubiquitin carrier proteins. The ubiquitin is then transferred to the substrate protein by an E3, an ubiquitin-protein ligase. Eukaryotic cells contain perhaps hundreds of E3 enzymes, and the specificity of the pathway is due to the ability of different E3s to recognize different degradation signals on the substrates. The resulting ubiquitinylated proteins are then recognized and degraded by a 2.4 MDa proteolytic complex, the 26S proteasome [27].

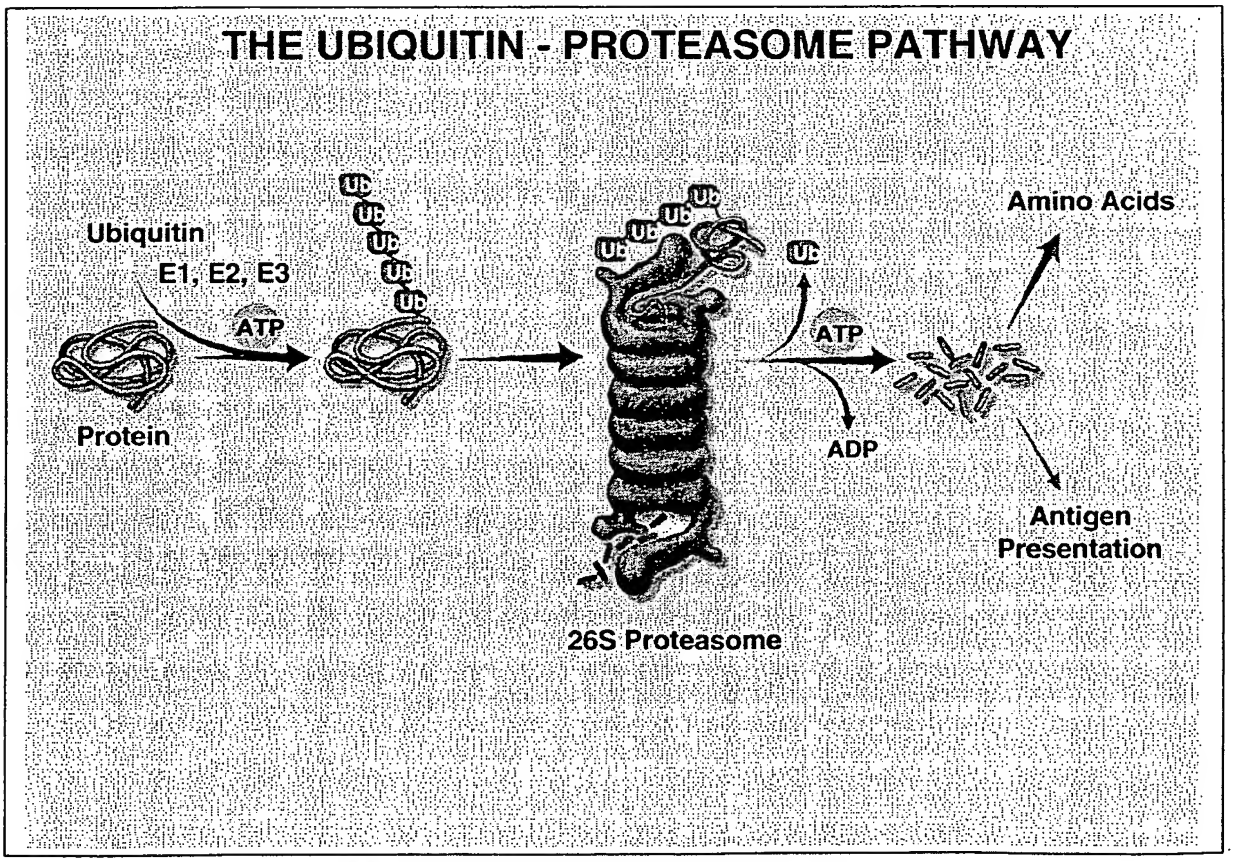

Fig. 1. The simplified scheme of the ubiquitin–proteasome pathway.

Table 2
Unique properties of 26S proteasome

- Enormous size (2.5 MDa), at least 44 polypeptides
- ATP-dependent
- Active sites segregated in secluded compartment
- Six proteolytically active sites with three different specificities
- Degrades proteins processively
- N-terminal threonine-based proteolytic mechanism
- Can unfold globular proteins
- Substrate recognition by polyubiquitin chain

2. 26S proteasome – a multifunctional proteolytic machine

The 26S proteasome is an ATP-dependent, multifunctional proteolytic complex that differs in many respects from typical proteolytic enzymes (Table 2). It consists of a proteolytic core particle, the 20S (720 kDa) proteasome, sandwiched between two 19S (890 kDa) 'cap' regulatory complexes [28], also termed PA700 (Fig. 2). These complexes associate together in an ATP-dependent manner [27,29]. Besides these 19S–20S–19S complexes, single-capped 19S–20S complexes are likely to exist in vivo [30,31], and it is unclear which of these forms is more important in protein breakdown. In certain tissues and cell types, single-capped complexes may also associate with the interferon-γ (IFN-γ)-induced heptameric ring PA28 (11S) complex [31], which is believed to stimulate production of antigenic peptides by proteasomes [32] by an unclear mechanism. In addition, free 20S proteasomes as well as symmetric PA28–20S–PA28 complexes exist in mammalian cells [30,31] but they are unable to degrade ubiquitin-conjugated proteins and are not ATP-dependent. Thus, they are unlikely to play a significant role in intracellular proteolysis, which in vivo is an ATP-dependent process [33].

The 20S proteasome is a hollow cylindrical particle consisting of four stacked rings (Fig. 3). Each outer ring is composed of seven different α subunits, while each inner ring is composed of seven different β subunits [34]. Each β-ring contains three different proteolytically active sites (Fig. 3), which were identified by X-ray diffraction as the sites of binding of a peptide aldehyde inhibitor [35]. All these active sites face the inner chamber of the cylinder, and the only way for substrates to reach this chamber is through the gated channels in the α-rings [35–37], which is too narrow to be traversed by tightly folded globular proteins [38]. Moreover, as demonstrated by X-ray diffraction [35], these channels are completely closed in the free latent 20S proteasomes. Thus, in contrast to the majority of other proteases which have easily accessible active sites, proteasomes have active sites which are confined to the inner cavity of the 20S core, thereby preventing uncontrolled destruction of the bulk of cellular proteins.

19S regulatory complexes control the access of substrates into the proteolytic core (Fig. 2). Each 19S particle consists of a base and a lid [27,39]. The lid, which contains

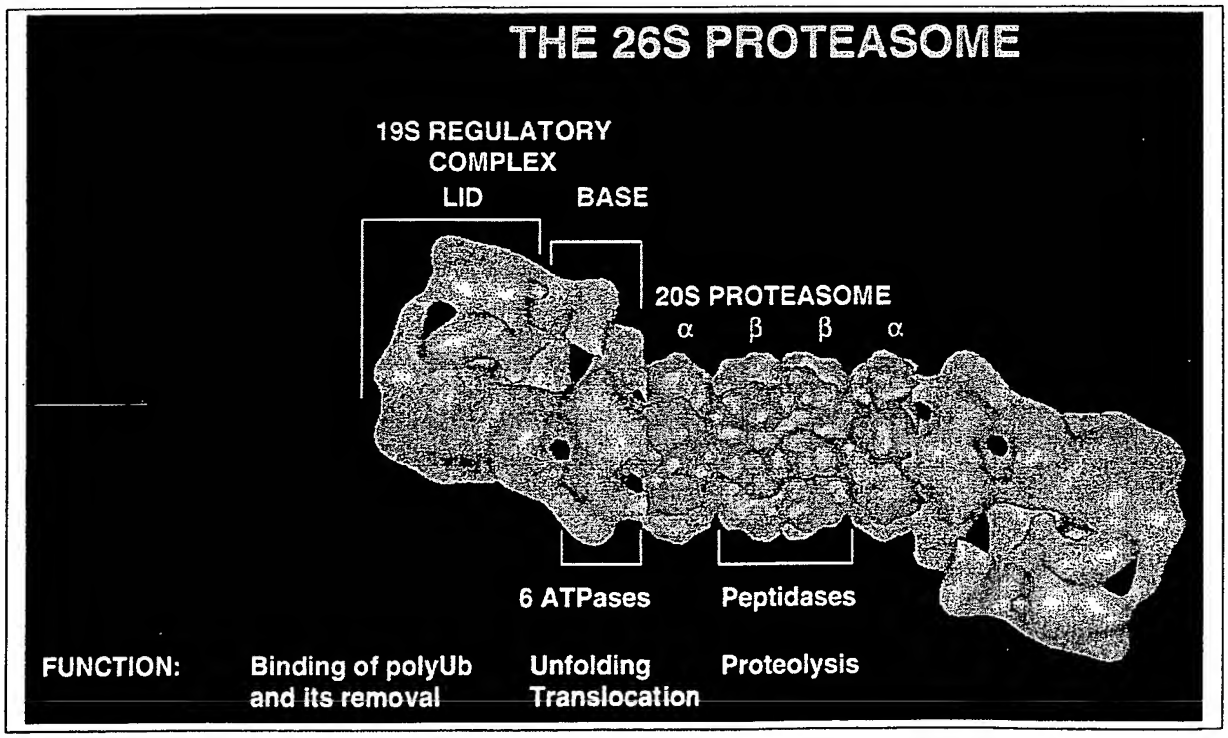

Fig. 2. The 26S proteasomes and its components. Electron tomography image [27] was kindly provided by W. Baumeister.

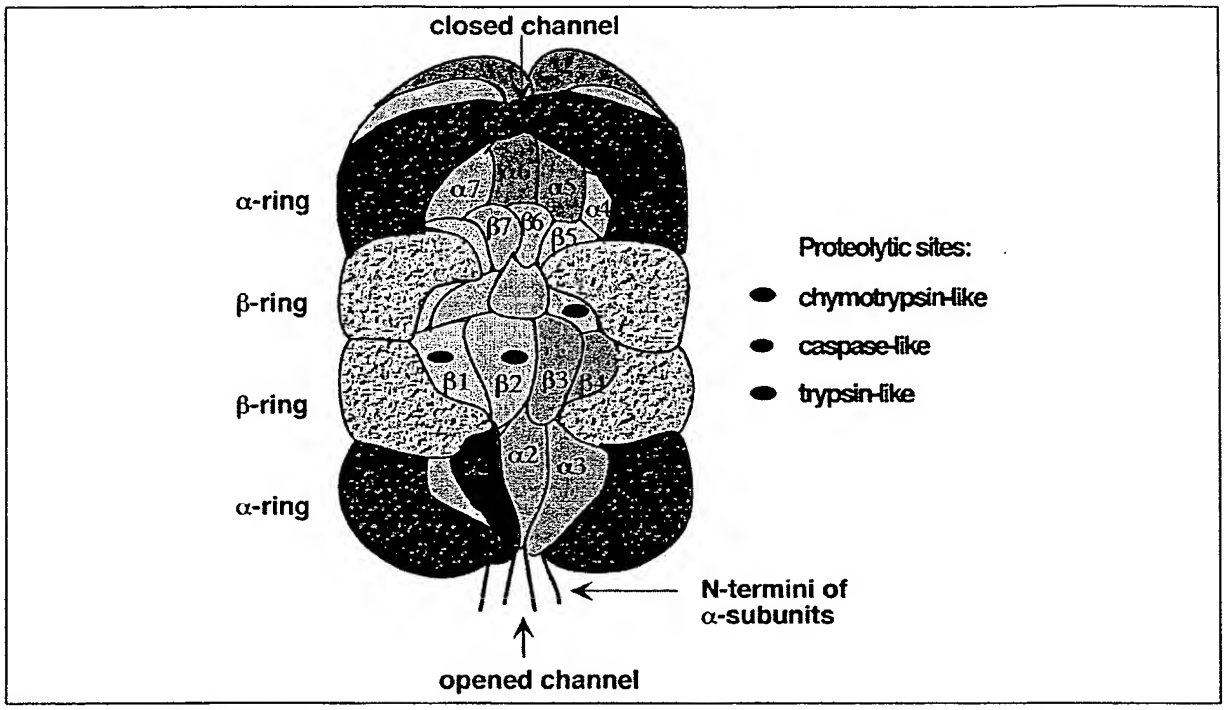

Fig. 3. The 20S proteasome. Schematic representation of the cross-section of the particle showing topology of the subunits and location of three active sites. The entrance channel on the top of the particle is shown in the closed conformation, while the channel on the bottom of the particle is in the open conformation. Location of the N-termini of α subunits, which are responsible for closing the channel, are shown according to the structure of yeast proteasome with PA26 activator from *Trypanosoma brucei*, which is the structural homologue of the mammalian PA28 activator [152]. The stippled areas represent the cut surfaces of the particle.

at least nine polypeptides, is believed to bind to the polyubiquitin chain with high affinity and to cleave it away from the substrate. The base, which associates with the 20S particle, consists of eight polypeptides including six homologous ATPases of the AAA family. As demonstrated by site-directed mutagenesis, these ATPases interact directly with α-rings of the 20S core particle resulting in the ATP-dependent opening of the channel in the α-rings [40], which allows polypeptides access into the proteolytic chamber of the 20S particle.

ATPases of the 19S complexes are also likely to unfold the polypeptide and catalyze its translocation into 20S proteasomes. In prokaryotes and archaea, which lack ubiquitin but contain simpler 20S proteasomes [41], proteasomes can be regulated by hexameric rings of ATPases homologous to the ATPases of the 19S complexes [42,43]. These ATPases have been shown to mediate protein unfolding [44] and facilitate entrance of substrates into the core proteasome.

Unlike the great majority of proteases which cleave the substrate once and then release the two fragments, proteasomes degrade proteins in a processive fashion [45–47]. They cut polypeptides at multiple sites without the release of polypeptide intermediates, generating peptides which range from three to 22 residues in length with a median size of six residues [46]. During this process the undegraded ubiquitin molecules are released by 19S particles for reuse in degradation of other substrates.

3. Active sites of eukaryotic 20S proteasomes

Although the 26S proteasome exhibits many different enzymatic activities, small molecule inhibitors are available only for the proteolytic sites of the 20S proteasomes. Eukaryotic core particles contain six active sites, three on each of its two central β-rings, and these proteolytic sites differ in their specificities (Fig. 3). Two termed 'chymotrypsin-like' cut preferably after hydrophobic residues and have their catalytic residues located on the β5 subunits. Two sites, located on β2 subunits, are 'trypsin-like' in cleaving after basic amino acids. The two remaining sites, located on β1 subunits, split peptide bonds preferentially after acidic residues [47,48]. These latter sites were traditionally termed 'peptidyl glutamyl peptide hydrolase' [49]; however, it has been found that they cleave after aspartic acid residues faster than after glutamates, and therefore it was suggested that these sites be called 'post-acidic' or 'caspase-like' [50]. (Caspases are intracellular cysteine proteases, involved in cytokine processing and apoptosis, which cleave proteins only after aspartates [51].) It should be borne in mind that these names are

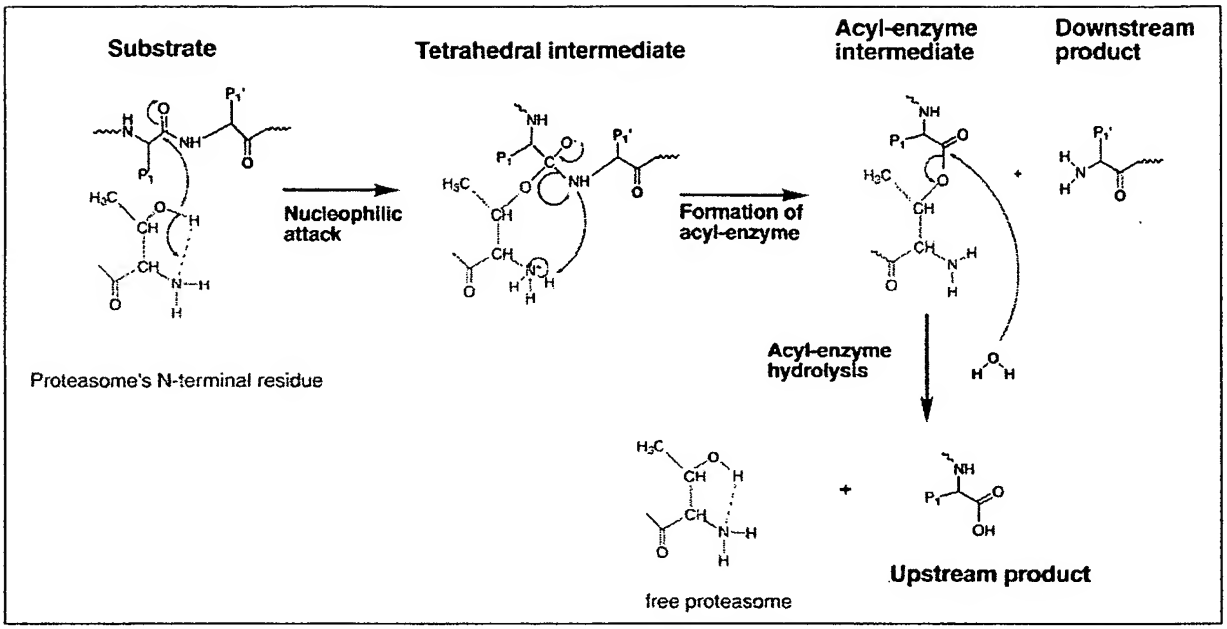

Fig. 4. Proteasome catalytic mechanism. Substrate is black, proteasome is cyan, and bonds formed during catalysis are blue.

imprecise and useful only to indicate their general similarities to the substrate specificities of 'classical' proteases, though they do not imply any similarity in catalytic mechanisms or physiological functions.

It has been claimed, based on studies with model peptides and inactivation by inhibitors, that mammalian proteasomes also contain two other peptidase activities, referred to as 'branched-chain amino acid-preferring' (cleaving after leucines) and 'small neutral amino acid-preferring' [52]. However, the existence of additional active sites responsible for these activities has been excluded by X-ray diffraction [35], extensive kinetic studies [53,54] and site-directed mutagenesis [48]. Surprisingly, the cleavages after branched-chain amino acids (i.e. leucine, isoleucine and valine) are performed mainly by post-acidic (caspase-like) sites [48,53,55], and to a lesser extent by the chymotrypsin-like sites [54]. Thus, the specificities of the proteasome's active sites are actually broader than reflected by their names.

Substrate binding sites for each of these catalytic β subunits are formed as a result of specific interactions of the catalytic subunit with one of its adjacent β subunits [35,36]. Consequently, subunits of the proteasome do not exhibit proteolytic activity when expressed individually and have not been detected in cells in the monomeric form. Thus, the 20S proteasome is not a complex of different individual proteases, but a unique multicatalytic enzyme whose multiple active sites function only as an integral part of this particle. Moreover, the activities of some of these peptidase sites can be allosterically stimulated or inhibited by peptide substrates acting on non-catalytic regulatory sites [50,56,57]. These findings thus suggest that function of different active sites in protein breakdown is a regulated or even an ordered process.

4. The proteasome's unusual catalytic mechanism

Proteasomes, together with their bacterial homologue HslVU complex [58], form a new class of proteolytic enzymes called threonine proteases. Unlike any other protease, all the proteolytic sites in proteasomes utilize N-terminal threonines of β subunits as the active site nucleophiles. The proteasome is a member of the growing group of N-terminal nucleophile hydrolases, which have similar 3D structure and utilize the side chains of their N-terminal serine, threonine or cysteine residues to cleave various amide bonds [59]. Much of our understanding of this unique proteolytic mechanism has come through studies using inhibitors and site-directed mutagenesis [48,60].

Peptide aldehydes are well-characterized inhibitors of two other classes of proteolytic enzymes, serine and cysteine proteases. They inhibit these enzymes reacting with the catalytic hydroxyl or thiol groups in the active sites of those enzymes to form a reversible hemi(thio)acetal, which resembles a transition state analogue of the enzymatic reaction [61]. Similarly, the peptide aldehyde Ac-Leu-Leu-nLeu-al (commonly termed ALLN, Fig. 6) was shown by X-ray diffraction to form a hemiacetal bond with the hydroxyl group of the N-terminal threonines of the proteasome's β subunits (Fig. 7), strongly suggesting that this hydroxyl is the catalytic nucleophile [35,36]. Accordingly, mutation of this threonine to alanine completely abolished the activity of the enzyme, while its mutation to serine

Table 3
Different classes of proteasome inhibitors and their selective representatives

Class	Compound	Inhibition of different active sites			LC_{80} in cells (μM)[a]	Other intracellular targets (IC_{50})
		Chymotrypsin-like	Trypsin-like	Caspase-like		
Reversible inhibitors		K_i (nM)				
Peptide aldehydes	MG132 (Z-LLL-al)	2[b]–4[e]	2760[b]	900[b]	0.4	Calpain, cathepsins
	PSI (Z-IE(OtBu)AL-al)	IC_{50} = 250 nM[d]	n.a.	n.a.	n.a.	Calpain (2.5 μM), cathepsins
	CEP1612	< 2[e]	⩾ 1000[e]	n.a.	n.a.	Calpain (100 nM), cathepsin B (90 nM)
Peptide boronates (slow on and off rates)	MG262 (Z-LLL-bor)	0.03[e]	n.a.	n.a.	0.04	Not tested
	PS341	0.62[e]	n.a.	n.a.	0.02	None found
	PS273 (MNLB)	0.15[f]	n.a.	n.a.	n.a.	Not tested
Irreversible inhibitors		k_{ass} (M^{-1} s^{-1})				
Lactacystin and derivatives	Lactacystin	194[g]	10[g]	4.2[g]	4	Cathepsin A, TPPII
	β-Lactone (omuralide)	8 500[h]	253[h]	37[h]	n.a.	Cathepsin A, TPPII
Peptide vinyl sulfones	NLVS (Nip-LLL-vs)	13 400[g]	422[g]	100[g]	8	Cathepsins S and B (k_{ass} = 190 M^{-1} s^{-1})
	YLVS (YLLL-vs)	1 500[g]	560[g]	20[g]	n.a.	Not tested
Peptide epoxyketones	Dihydroeponemycin	65[i]	4.4[i]	61[i]	n.a.	Cathepsin B (very weak)
	Epoxomicin	37 000[i]	79[i]	37[i]	0.03	None found
	YU101 (Ac-hFLFL-ex)	166 000[j]	7.1[j]	21[j]	0.25	Not tested

[a]LC_{80} is inhibitor concentration resulting in 80% growth inhibition of EL4 cells [125]. Other values in the table are from: [b]T. Akopian, unpublished observations; [c][73]; [d][77]; [e][74]; [f][139]; [g][88]; [h][12]; [i][92]; [j][148]

retained significant activity [60,62]. In addition, this threonine residue is covalently modified by different inhibitors, 3,4-dichloroisocoumarin [45,63] (DCI, Fig. 6), lactacystin [64], vinyl sulfones [65], and epoxyketones [12] (Figs. 5 and 7, see below).

The sensitivity of proteasomes to peptide aldehydes strongly suggests that its hydrolytic mechanism resembles the well-characterized mechanism of serine and cysteine proteases, even though the proteasome lacks the classical catalytic triad of these enzymes [36]. Instead, the free N-terminal amino group of catalytic threonine is best positioned to accept the proton from the side chain hydroxyl, which is transferred either directly or shuttled by a water molecule [35]. The pK_a of the N-terminal amino group may be lowered by the side chain amino group of the adjacent conserved Lys-33, which is essential for catalysis [60,66,67].

The other features of the proteasome's catalytic mechanism must be similar to those of serine proteases. First, the hydroxyl group of the proteasome's catalytic threonine directly attacks a scissile bond (Fig. 4). This attack results in the formation of the tetrahedral intermediate, which then collapses into an acyl enzyme with the release of the first reaction product. Deacylation of catalytic threonine residues by water leads to the formation of the second product and the regeneration of free proteasomes. Although an acyl enzyme intermediate has not been isolated for the proteasome, the demonstration by X-ray diffraction that the catalytic hydroxyl is acylated by the β-lactone inhibitor (see below, Fig. 8) provides strong evidence for its existence [35].

5. Major classes of proteasome inhibitors

Enzyme-specific inhibitors of proteases are usually short peptides linked to a pharmacophore, generally located at its C-terminus. The pharmacophore interacts with a catalytic residue with the formation of reversible or irreversible covalent adduct, while the peptide portion specifically associates with the enzyme's substrate binding pocket in the active site. Although the proteasome has multiple active sites, inhibition of all of them is not required to significantly reduce protein breakdown. In fact, inhibition of the chymotrypsin-like site or its inactivation by mutation alone causes a large reduction in the rates of protein breakdown [5,66,67]. In contrast, inactivation of trypsin-like or caspase-like sites had little effect on overall proteolysis [46,67,68]. In addition, most inhibitors of chymotrypsin-like sites are highly hydrophobic and consequently much more cell-permeable than inhibitors of the trypsin- or caspase-like sites, which contain charged residues. Consequently, almost all the synthetic and natural inhibitors of the proteasome act predominantly on the chymotrypsin-like activity but also have some, usually much weaker, effects on the two other sites.

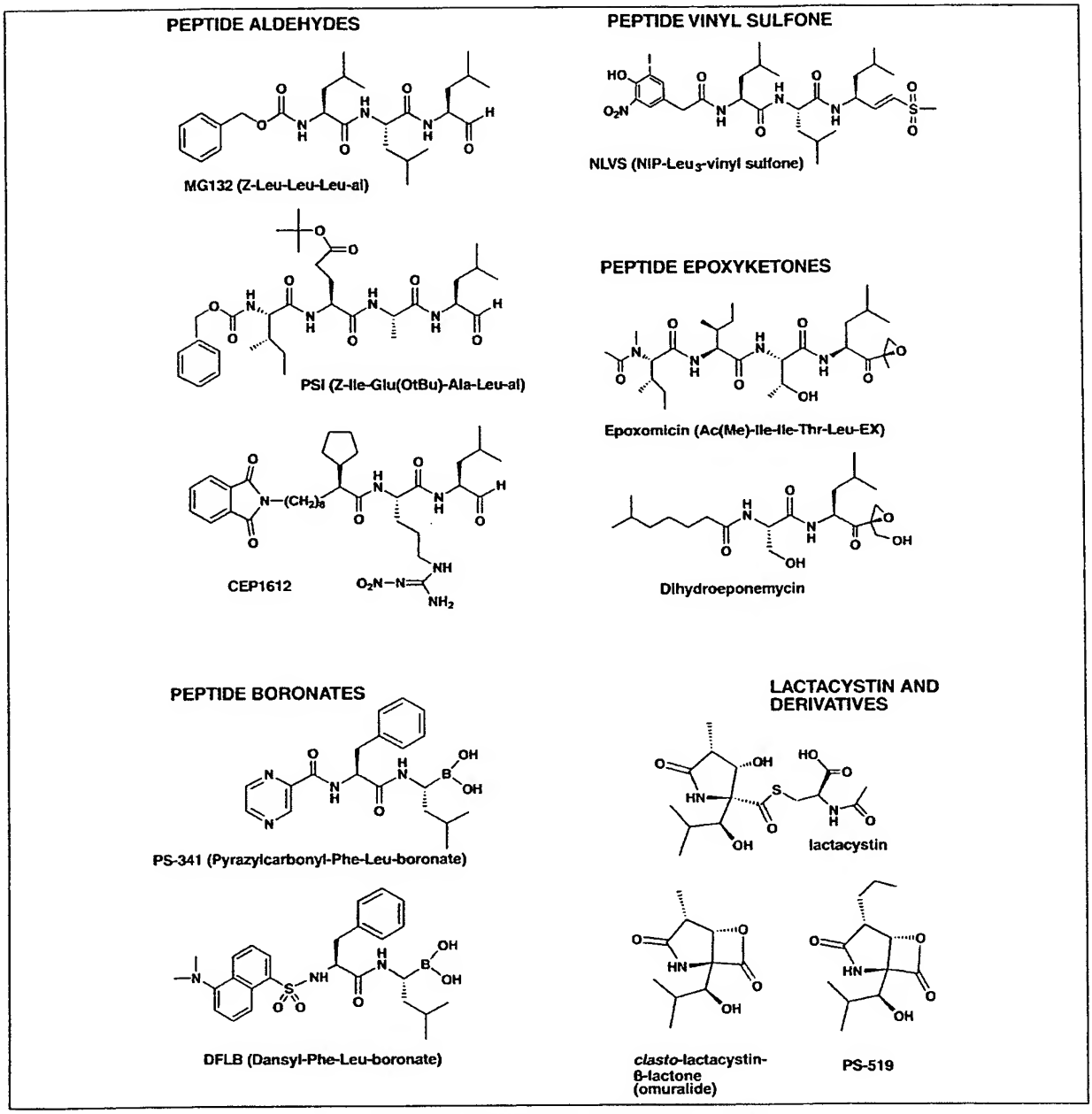

Fig. 5. Major proteasome inhibitors. Pharmacophores are red.

Although cleavages by the chymotrypsin-like sites appear to be rate-limiting in protein breakdown, the extent of inhibition of chymotrypsin-like activity cannot be directly related to the reduction in protein breakdown because inhibition of this site may lead to the enhanced cleavages by two other sites. Unfortunately, inhibitor potency has often been measured only against the chymotrypsin-like activity with purified proteasomes and synthetic peptide substrates. The various reports concerning the potencies of different proteasome inhibitors in cells are difficult to compare because different investigators have used diverse cell lines and assays, which were generally only indirectly related to the rates of protein breakdown. Thus, when comparing different inhibitors, in this review we will use indicated degree of inhibition of chymotrypsin-like activity, even though biological efficacy should involve capacity to inhibit degradation of whole proteins.

The chymotrypsin-like site of proteasomes cleaves primarily after large hydrophobic residues, similar to the preference of intracellular cysteine proteases such as cyto-

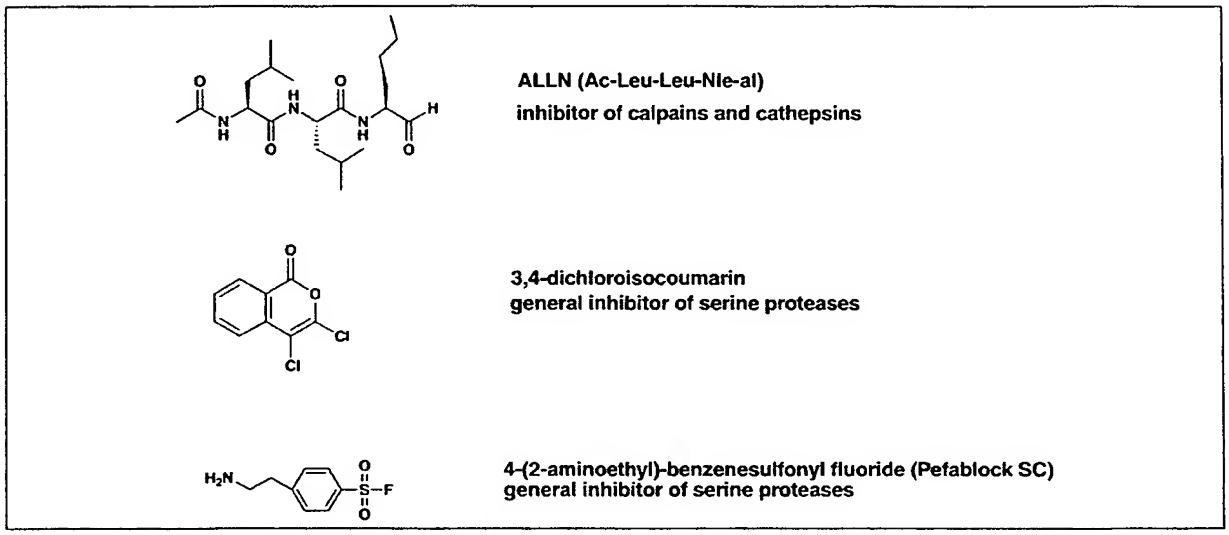

Fig. 6. Non-selective inhibitors of proteasome. Pharmacophores are red.

solic calpains and many lysosomal cathepsins [69]. Therefore, high selectivity of proteasome inhibition by peptide-based compounds would be hard to achieve just by simply manipulating the peptide portion of the inhibitor. Instead, the use of a pharmacophore with preference for the proteasome's N-terminal threonine is required. Based on pharmacophores, proteasome inhibitors can be divided into several groups.

5.1. Peptide aldehydes

Peptide aldehydes were the first proteasome inhibitors to be developed [5,70] and are still the most widely used inhibitors. Aldehyde inhibitors of the chymotrypsin-like site are slow-binding [70], but they enter cells rapidly and are reversible. These inhibitors have fast dissociation rates, are rapidly oxidized into inactive acids by cells and are transported out of cell by the multi-drug resistance (MDR) system carrier (J. Adams, personal communication). Consequently, in experiments involving cultured mammalian cells and yeast, effects of these inhibitors can be rapidly reversed by removal of the inhibitor [71].

As discussed above, peptide aldehydes are well-known inhibitors of cysteine and serine proteases, and thus can inhibit such proteases in vivo. For example, ALLN, which was used in earlier studies, was first described as a calpain inhibitor I [72], and is 25-fold more potent against cathepsin B and calpain than the proteasome [5]. Many other peptide aldehydes have been synthesized since [73,74], but only a few of them are now used widely. MG132 (Z-Leu-Leu-Leu-al, also termed Cbz-LLL or z-LLL, Table 3 and Fig. 5) is not only significantly more potent than ALLN against the proteasome [75], but is much more selective, as shown by the fact that inhibition of calpains and cathepsins requires at least 10-fold higher concentrations [76]. Another peptide aldehyde, PSI (Z-Ile-Glu(Ot-Bu)-Ala-Leu-al, Fig. 5), inhibits the proteasome 10-fold better than calpain but is less potent than MG132 [77] (Table 3). Finally, the dipeptide aldehyde CEP1612 (Fig. 5) appears at least as good as MG132 in potency and selectivity [74] (Table 3), but is not available commercially.

Since MG132, PSI, MG115 (Z-Leu-Leu-nVal-al) and ALLN can all inhibit calpains and various lysosomal cathepsins in addition to the proteasome, when using these inhibitors in cell culture it is important to perform control experiments to confirm that the observed effects are due to the inhibition of the proteasome. First, one can use agents, which block intracellular cysteine proteases, but do not inhibit proteasomes [5]. Such inhibitors are Z-Leu-Leu-al, and E-64 for calpains [76], and weak bases such as chloroquine and E-64 for lysosomal proteolysis [5]. In yeast, where digestive vacuoles contain mainly serine, not cysteine, proteases, phenylmethylsulfonyl fluoride can be used to inhibit these enzymes without affecting proteasomes [71]. Moreover, with any effect sensitive to MG132 or other aldehydes, involvement of the proteasome can be confirmed [6] or ruled out [78] by use of several more specific inhibitors of the proteasome, such as lactacystin, epoxomicin and boronate MG262 (Fig. 5 and Table 3, see below), which would be too expensive for most investigators to use in routine studies.

Despite the availability of these inhibitors, MG132, due to its low cost and the rapid reversibility of its action, still remains, in our opinion, the first choice to study proteasome involvement in a process in cell cultures or tissues, if appropriate controls are used. As the most potent and selective of commercially available aldehydes, MG132 is preferable to ALLN, MG115 (Z-Leu-Leu-nVal-al), or even PSI. On the other hand, the least selective inhibitor, ALLN, because of its ability to inhibit most major pro-

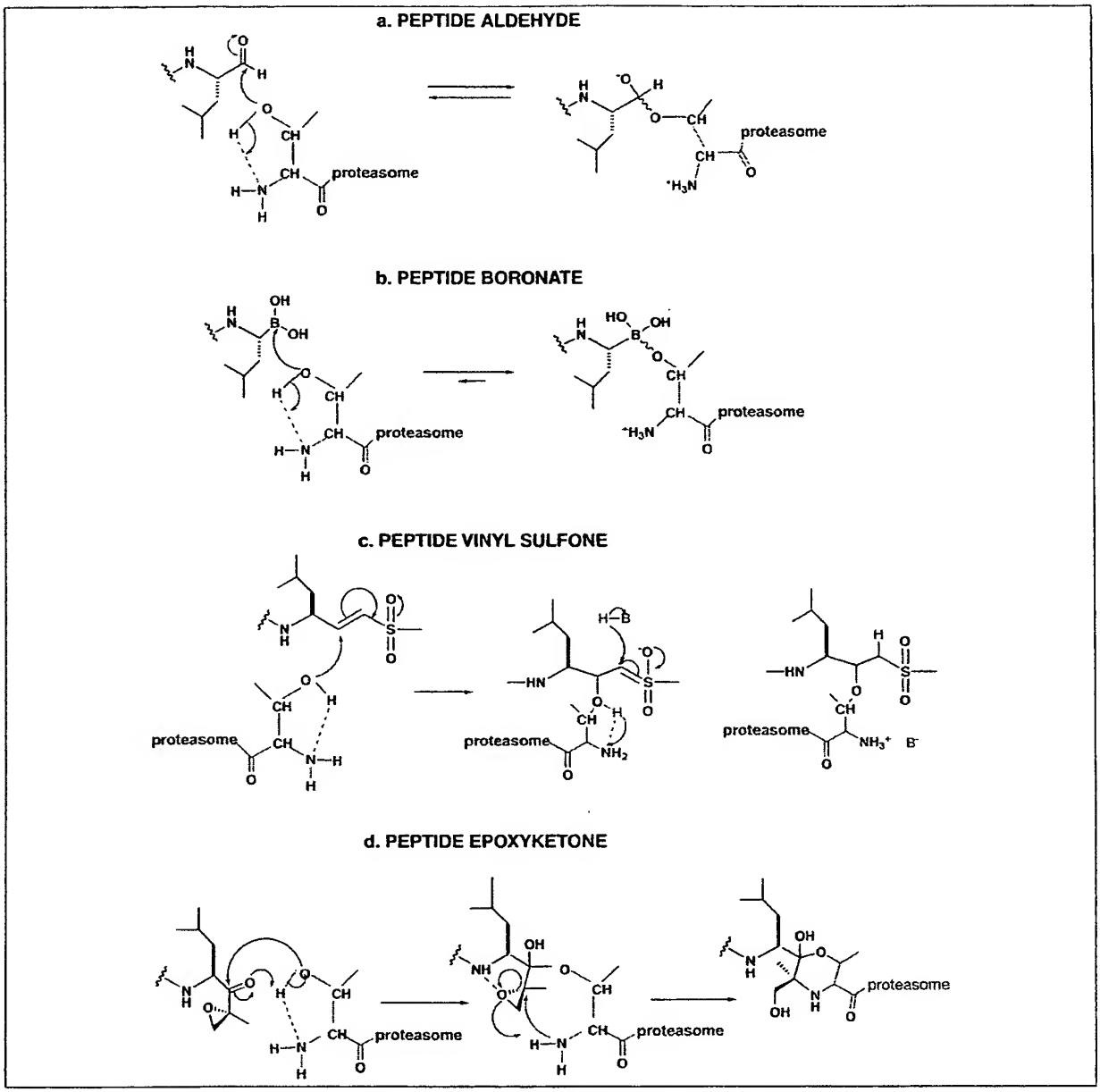

Fig. 7. Mechanisms of proteasome inhibition by different classes of peptide-derived inhibitors. Inhibitors are in black, except pharmacophores, which are in red. Proteasome is in cyan, and newly formed bonds are blue.

teases in mammalian cells, is probably the best tool for prevention of unwanted proteolysis, for example during isolation of proteins from mammalian cells.

5.2. *Peptide boronates*

Peptide boronates (Table 3 and Fig. 5) are much more potent inhibitors of the proteasome than the aldehydes [73]. The boronate analogue of MG132, MG262 (Z-Leu-Leu-Leu-boronate), is 100-fold more potent than the aldehyde with an impressive K_i of 18 pM [73], and even some dipeptide boronates have K_i values below 1 nM (Table 3). The mechanism of inhibition by these slow-binding compounds is yet to be confirmed by X-ray diffraction, but it is presumed that boronates, like peptide aldehydes, form a tetrahedral adduct with the active site threonine (Fig. 7). The boronate–proteasome adducts have much slower dissociation rates than proteasome–aldehyde adducts, and although boronates are considered reversible inhibitors, the inhibition is practically irreversible over hours.

Boronates are also more selective inhibitors than aldehydes. Unlike aldehydes and vinyl sulfones (see below),

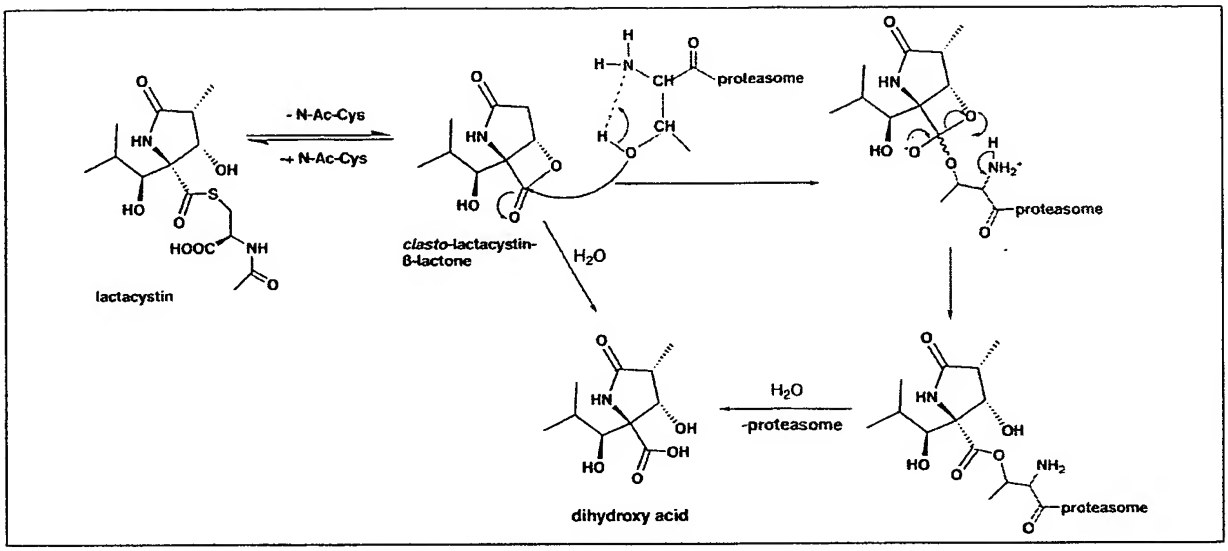

Fig. 8. Mechanism of proteasome inhibition by lactacystin and its β-lactone.

boronates are very poor inhibitors of thiol proteases, due to the weak interactions between sulfur and boron [73]. Inhibition of serine proteases by many peptide boronates, such as PS-341 (pyrazylcarbonyl-Phe-Leu-boronate, Fig. 5), is also 1000-fold weaker than that of the proteasome [73]. An extensive search failed to identify any other targets of PS-341, aside from the proteasome, in the rat [79]. Boronates, unlike aldehydes, are not inactivated by oxidation and are not rapidly secreted from cells by MDR (J. Adams, personal communication).

This combination of potency, selectivity and metabolic stability makes the peptide boronates (originally developed at ProScript and now being developed by Millennium Pharmaceuticals), better drug candidates than other classes of proteasome inhibitors, and one of the dipeptide boronates, PS-341, is currently in phase II clinical trials in cancer patients (see below). Two other dipeptide boronates, dansyl-Phe-Leu-boronate (DFLB, Fig. 5) and PS-273 (morpholino-naphthylalanine-Leu-boronate, also termed MNLB or MG273), are useful fluorescent probes of the active sites, because binding of these inhibitors to them enhances the fluorescence of the environment-sensitive dansyl and naphthyl moieties [80].

5.3. Non-peptide inhibitors: lactacystin and β-lactone

Lactacystin is a *Streptomyces* metabolite, which was discovered by Omura and coworkers as a result of its ability to induce differentiation of cultured neuronal cells [81] and was later synthesized by Corey and coworkers [82]. In studies of its mode of action, Fenteany et al. [64] found that radiolabeled synthetic lactacystin selectively modified the β5(X) subunit of mammalian proteasome and irreversibly blocked its activity. Other proteolytic sites of the proteasome were also modified and reversibly inhibited but at much slower rates [6,64]. Subsequent studies, however, demonstrated that lactacystin itself is not active against proteasomes in vitro but, at neutral pH, undergoes spontaneous transition to *clasto*-lactacystin-β-lactone [83] (Figs. 5 and 8), for which the name 'omuralide' was suggested [82]. Kinetic analysis demonstrated that this β-lactone, but not lactacystin, reacts with the proteasome's active site threonines [83], resulting in the opening of the β-lactone ring and acylation of the proteasome's catalytic hydroxyl (Fig. 8). The structure of the lactacystin–proteasome complex was determined by X-ray diffraction, and provided strong evidence that an acyl enzyme conjugate is indeed an intermediate in catalysis by the proteasome [35].

Plasma membranes of mammalian cells [84] and yeast [71] are not permeable to lactacystin, but the β-lactone, which is spontaneously formed in mammalian tissue culture media, easily enters cells [84] (Fig. 8). In yeast, lactacystin is not active [71], probably because lactonization does not occur in acidic media. The β-lactone is the least stable of all proteasome inhibitors. It is rapidly hydrolyzed by water at neutral pH and exists inside mammalian cells in equilibrium with lactathione, an inactive product of its reaction with glutathione [84]. In addition, although the β-lactone is considered an irreversible inhibitor, its adduct with proteasome is slowly ($t_{1/2} \approx 20$ h) hydrolyzed by water, resulting in the recovery of proteolytic activity. Replacement of the catalytic threonine by serine in archaeal proteasomes increases the rate of inactivation of proteolytic activity and markedly increases the rate of hydrolysis of this acyl enzyme [62], perhaps explaining why this β-lactone does not inhibit most serine proteases. The only serine proteases known to be inhibited by the β-lactone are cathepsin A, a lysosomal carboxypeptidase [85], and cytosolic tripeptidyl peptidase II [86]. Thus, the β-lactone is a

significantly more selective proteasome inhibitor than peptide aldehydes.

5.4. DCI

DCI, a broad irreversible inhibitor of serine proteases [61] (Fig. 6), also inhibits proteasomes by covalent modification of its N-terminal threonine [45,63]. The exact mechanism remains unknown, but like β-lactone, DCI contains a cyclical ester, suggesting that it inhibits the proteasome by the formation of non-hydrolyzable acyl enzyme. This compound is not useful for in vivo studies due to its toxicity and lack of selectivity.

5.5. Peptide vinyl sulfones

Peptide vinyl sulfones are synthetic irreversible inhibitors of proteasome first described by Bogyo et al. [65] that covalently modify its catalytic β subunits (Table 3 and Fig. 5). The structure of the resulting covalent adduct is currently being determined by X-ray diffraction (M. Groll, personal communication). It seems most likely that the hydroxyl group of the proteasome's catalytic threonine reacts with the double bond of the vinyl sulfone moiety in a Michael addition (Fig. 7), since mutation of this threonine to an alanine prevents modification of the β subunit [65].

Peptide vinyl sulfones do not inhibit serine proteases, but they were first described as inhibitors of cysteine proteases [87], and therefore the selectivity of inhibition depends on the peptide portion of the inhibitor. For example, replacement of the benzyloxycarbonyl (Z) group in ZLVS (Z-Leu_3-VS), a vinyl sulfone analogue of MG132, by the 3-nitro-4-hydroxy-5-iodophenylacetate (NIP) group to generate NLVS (Fig. 5) significantly reduced the inhibition of cathepsins B and S [65].

Vinyl sulfones are easier to synthesize than other irreversible inhibitors of the proteasome, and an advantage of such covalent inhibitors is that they can be used as sensitive active site probes [65] for mechanistic studies of proteasomes in different tissues and cells. Several vinyl sulfones containing tyrosine or a nitrophenyl group were synthesized to allow easy radioiodination. Incubation of cellular extracts with such compounds results in the covalent attachment of the radiolabeled compounds to the active subunits of the proteasome, which can then be identified by comparing the mobility of radioactive spots on 2D gels with the known pattern of proteasomal subunits on 2D gels. Two compounds most widely used as probes are NLVS, which modifies predominantly the chymotrypsin-like subunits, and [^{125}I]YL_3VS (Tyr-Leu-Leu-Leu-VS), which reacts with all subunits, although at different rates [88,89]. Most recently, the compounds NIP-Leu-Leu-Asn-VS [90] and Ada-[^{125}I]Tyr-Ahx_3-Leu_3-VS, which react with all three active sites at comparable rates, and a biotinylated analogue of one of them, Ada-Lys(Bio)-Ahx_3-Leu_3-VS, have been synthesized [91].

5.6. Epoxyketones

A major recent development was the discovery that natural epoxyketones (α′,β′-ketoepoxides), such as epoxomicin and eponemycin (Fig. 5), isolated based on their antitumor activity in mice, exert their biological effects by proteasome inhibition [12,92]. Epoxomicin reacts primarily with the chymotrypsin-like site, while the less potent epoxyketone eponemycin and its synthetic analogue dihydroeponemycin (Fig. 5) react with the caspase-like and chymotrypsin-like sites at similar rates (Table 3). These compounds act by an interesting and unique mechanism, in which they react with both the hydroxyl and amino groups of the catalytic N-terminal threonine of the proteasome (Fig. 7). The crystal structure of the yeast proteasome in complex with epoxomicin revealed a six-membered morpholine ring, formed by the N-terminal threonine and epoxyketone moiety of the inhibitor [93]. This structure suggests that the catalytic hydroxyl first attacks the carbonyl group of the pharmacophore (Fig. 7). Then, the free α-amino group of the threonine opens up the epoxy ring and completes the formation of the morpholino adduct [93]. Biotinylated derivatives of epoxomicin and eponemycin were also prepared and these inhibitors, like vinyl sulfones, can be used as active site probes.

Epoxyketones, because of their unique mechanism, are the most selective inhibitors of the proteasome known. Indeed, proteasomal subunits are the only cellular proteins covalently modified by the biotinylated derivatives of epoxomicin and eponemycin [12,92]. In addition, epoxomicin, in contrast to the homologous aldehyde, did not inhibit any other proteolytic enzyme tested [12]. However, the possibility that these epoxyketones may reversibly inhibit other enzymes has not been studied.

The reason for this high specificity of epoxyketones most likely lies in their ability, in 2(*R*) configuration, to take specific advantage of the proteasome's unique catalytic mechanism by forming a cyclical morpholino ring (Fig. 7). Epoxyketones cannot form such a ring with a cysteine or a serine protease, because these enzymes do not have a free N-terminus adjacent to the nucleophilic group [93]. Although some epoxides, for example E-64, inhibit cysteine proteases, they have a second electrophilic carbonyl group adjacent to the pharmacophore and (*S*) configuration at the C2 atom of the epoxy ring, while in the proteasome inhibitors this atom has the (*R*) configuration [93]. Inverting the configuration from *R* to *S* dramatically decreased the potency of proteasome inhibition [12], probably because the formation of the morpholine ring was sterically hindered in 2(*S*) epimers [93].

5.7. Other classes of synthetic proteasome inhibitors

Other classes of synthetic proteasome inhibitors have been reported. These include reversible peptide α-ketoaldehydes [94] and α-ketoamides [95], which are similar to aldehydes in potency and selectivity, and indanone peptides [96], which are even less potent. Thus, these compounds do not appear to offer any advantage over other classes of inhibitors.

5.8. Bivalent inhibitors

Bivalent inhibitors of the proteasome were generated by tethering two peptide aldehydes with a flexible polyethyleneglycol (PEG) linker, long enough to span the distance between two active sites [97]. Although the resulting inhibitors were 100-fold more potent that their monovalent analogues, it is very unlikely that these PEG-tethered compounds will be cell-permeable. However, these agents or analogous bivalent inhibitors with different pharmacophores may be useful tools for certain studies on the purified particles.

5.9. New natural compound inhibitors

Recently several agents capable of inhibiting the proteasome have been found among natural compounds (Fig. 9). TMC-95A, a cyclic peptide metabolite of *Apiospora montagnei*, is a potent competitive inhibitor of the chymotrypsin-like activity of pure 20S proteasomes ($K_i = 2.3$ nM), but also inhibits caspase-like and trypsin-like activities [98]. In contrast to other inhibitors, whose mode of inhibition was studied by X-ray diffraction, TMC-95A does not modify catalytic threonine but binds to the active site by an array of hydrogen bonds between inhibitor and enzyme active sites [99]. At low micromolar concentrations, this compound showed cytotoxicity against cancer cell lines, but it is not yet clear whether this toxicity is due to the inhibition of protein breakdown.

Gliotoxin (Fig. 9) is fungal metabolite, which inhibits proteasomes by an unusual mechanism [100]. In vitro this non-peptide cyclic compound allosterically inhibits the chymotrypsin-like activity of 20S proteasome by binding to an unknown non-catalytic site, whereas all other inhibitors bind at the proteolytic sites. The disulfide bridge in gliotoxin is essential for its activity, suggesting that it acts by forming mixed disulfide bond with a proteasomal subunit. In cell culture, gliotoxin completely blocks degradation of IκB and other proteasome substrates, but at almost 100-fold lower concentrations than required for similar inhibition of purified proteasomes. Therefore, it remains to be proved whether proteolytic activities of the 20S proteasome are indeed the main targets of this inhibitor in vivo.

Green tea contains many cathechin-3-gallate derivatives. Several of these polyphenol compounds, especially (−)-epigallocatechin-3-gallate (EGCG) (Fig. 9), were found to be potent inhibitors of proteasome peptidase activities [101]. EGCG also inhibited degradation of several substrates of the pathway in cell culture and arrested cells in G_1 phase of the cell cycle. Structure–activity relationship studies, atomic orbital energy analysis and analysis of the products of EGCG interaction with proteasome strongly suggested that the ester bond in this molecule is attacked by the proteasome leading to the acylation of the reactive site threonine. The mechanism of proteasome inhibition by EGCG thus resembles that of lactacystin-β-lactone. Analysis of the reaction products suggests also that, like the proteasome-β-lactone adduct, proteasome-gallic acid acyl enzyme adduct is slowly hydrolyzed by water, leading to the reactivation of the proteasome.

5.10. Proteasome as the secondary target of different drugs

Finally, the chymotrypsin-like activity of the proteasome can also be inhibited by several major drugs, which were all developed as inhibitors of other enzymes. These include ritonavir, an inhibitor of the HIV-encoded aspartic protease [102], the lactonized pro-drug form of the HMG-CoA reductase inhibitor lovastatin [103], the anti-cancer DNA-intercalating agent aclacinomicin A (aclarubicin) [104], and the immunosuppressive agent cyclosporin A [105]. Although it is not clear whether these drugs have any effect on protein breakdown in vivo at their therapeu-

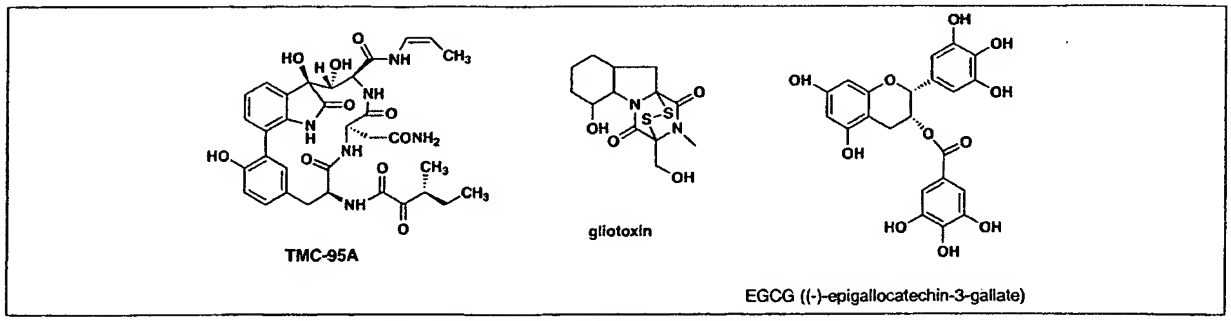

Fig. 9. New natural product inhibitors of the proteasome.

tic concentrations, these examples emphasize that any statement about the specificity of small molecules, based solely on in vitro studies, may not hold true in vivo.

6. Biological effects of proteasome inhibitors

Inhibitors of the proteasome have been essential tools in the discovery of many new substrates of the ubiquitin–proteasome pathway and establishing its role in different biological processes. Because of the importance of proteasome in many aspects of cellular function, its inhibitors have multiple, profound and often complex effects on living cells.

6.1. Inhibition of protein breakdown and its measurement in cells

The primary, immediate consequence of proteasome inhibition is a decrease of overall rates of protein breakdown in cells [5,6]. The concentrations of inhibitors required to cause such effects vary with the cell line [6]. The measurement of inhibitor efficacy in cells or extracts can be a major experimental challenge, and a number of the approaches used to measure efficacy of inhibitors in vivo are, at best, qualitative guides to inhibitor potency. As noted above, the degree of inhibition of the chymotrypsin-like activity is a useful qualitative measure of efficacy against proteins, but it does not indicate the actual reduction in degradation of proteins, especially if the inhibitor also affects the activity of the proteasome's other sites. By labeling cell proteins with radioactive amino acids, and then following the overall rates of breakdown of the radiolabeled species, it is possible to directly measure the total rate of intracellular proteolysis [33]. This approach has been used to measure the actual in vivo inhibition of protein breakdown by certain peptide aldehydes and lactacystin [5,6,71]). However, for many commonly used inhibitors (e.g. epoxomicin or vinyl sulfones), the capacity to reduce the overall rate of protein degradation in cells has still never been reported. The decay curves of radiolabeled cell proteins are complex exponential functions that initially reflect the hydrolysis of many short-lived proteins (half-life shorter than 2 h). With time, these curves reflect presumably the breakdown of long-lived proteins, whose half-lives range from hours to days. It is also possible in principle to follow the exponential breakdown of individual proteins (usually by immunoprecipitation) after either radiolabeling or blocking protein synthesis.

Several groups have attempted to quantitate inhibitor efficacy by measuring the accumulation of short-lived proteins. However, it should be noted that this approach does not reflect the degree of inhibition of proteolysis, since the buildup of a protein is determined both by its rate of synthesis and by its degradation rate. Also, since degradation is an exponential process, the actual effects on protein levels also depend on the protein's initial content and the number of half-lives over which this process is measured. Finally, in any period (e.g. several hours) an inhibitor would be expected to have a much larger effect on the levels of a short-lived protein than on a long-lived cell protein. For example, in the absence of synthesis, a 50% inhibition of proteolysis should double in 1 h the level of a protein with a half-life of 1 h, but should have a negligible effect on the levels of a protein with a half-life of 1 day (the average half-life of proteins in cell culture).

6.2. Induction of heat shock response

A decrease of overall rates of protein breakdown in cells [5,6] leads to rapid accumulation of short-lived proteins conjugated to ubiquitin [22,77,106]. Proteasome inhibition also causes an accumulation of misfolded and damaged proteins, which may constitute a large fraction (up to one-third) of newly synthesized polypeptides [22]. This accumulation of unfolded polypeptides triggers expression of heat shock proteins, stress proteins of the endoplasmic reticulum and, in yeast, accumulation of the thermoprotective disaccharide trehalose [107,108]. Consequently, even a short exposure (30–60 min) of cells to proteasome inhibitors protects them against various kinds of toxic conditions, such as increased temperature or oxygen radicals [108]. Transcriptional profiling with DNA microarray analysis of human cancer cells exposed to proteasome inhibitor confirmed the induction of heat shock genes, genes encoding proteasome subunits, as well as enzymes of amino acid and polysaccharide metabolism and a number of unknown genes [109]. Thus, inhibition of proteasome leads to a large number of changes in the cell.

6.3. Cytotoxicity of proteasome inhibitors

Although short-term exposure to proteasome inhibitors protects cells from a variety of toxic stimuli, long-term exposure to these compounds is toxic to nearly all cells and causes death by apoptosis. However, the relation between the degree and the duration of the reduction in proteolysis and apoptosis is unclear as the concentration of inhibitors [110,111] and length of exposure leading to cell death varies between cell types. Several studies demonstrated that proliferating cell lines are usually more sensitive to proteasome inhibitors than non-proliferating ones [112–116], and may undergo apoptosis within 4–48 h after addition of the inhibitor [112,117–119].

This greater sensitivity of proliferating cells to apoptosis may be a consequence of cell cycle arrest caused by the inhibition of the proteasome [79,111,120–122], as the proteasome is essential for the progression through many steps in the cell cycle [7]. Another possible reason for the higher toxicity of proteasome inhibitors in proliferating cells could be that these cells are more efficient in uptake, or slower in inactivating these compounds. Induc-

tion of apoptosis by proteasome inhibitors in certain cells has been attributed to the stabilization of the tumor suppressor protein p53 [117,121], a short-lived protein degraded by the proteasomes, whose accumulation can trigger the cell death program. However, proteasome inhibitors can also cause apoptosis in cell lines lacking p53 [79,110]. In addition, many other critical regulatory proteins, for example the cdk inhibitor p27 [11] or the proapoptotic protein bax [123], are stabilized, and various abnormal proteins accumulate when proteasome is inhibited. In fact, the build-up of such unfolded proteins alone can activate the stress kinase c-Jun N-terminal kinase (JNK), which can turn on the apoptotic cascade [118]. Inhibition of proteasome also prevents activation of NF-κB, which is a potent inhibitor of apoptosis [124]. Thus, toxicity of proteasome inhibitors is probably due to multiple factors, including simply the changes in normal protein composition of the cell, which must disturb homeostasis.

Recently, the cytotoxicity of several different inhibitors has been compared in EL4 cells in culture. Princiotta et al. [125] found that PS-341 is the most potent inhibitor, followed closely by epoxomicin and MG262. MG132 was 10-fold less potent that its boronate analogue (MG262). Lactacystin had to be present in 10-fold higher concentrations than MG132 to achieve the same effect, and NLVS was half as potent as lactacystin (Table 3).

In striking contrast to the effects on proliferating cells, less than 16 h treatment by proteasome inhibitors makes non-proliferating thymocytes and neuronal cell lines more resistant to apoptosis induced by ionizing radiation, glucocorticoids, phorbol ester, etoposide or deprivation of nerve growth factor [126–128]. Apoptosis may be temporarily inhibited because of the stabilization of the inhibitors of apoptosis bcl-2 and IAPs, which are degraded by the proteasome in response to certain apoptotic stimuli [129,130]. However, longer exposures of thymocytes to inhibitors eventually lead to death by apoptosis.

6.4. *Anti-tumor effects of proteasome inhibitors*

The ability of proteasome inhibitors to inhibit cell proliferation and selectively induce apoptosis in proliferating cells, together with their ability to inhibit angiogenesis [113,131], makes these agents attractive candidates as anti-cancer drugs. In addition, certain cancer cells require growth factors such as interleukin (IL) 4 and IL-6, whose production depends on NF-κB, and NF-κB activation is blocked by proteasome inhibitors [75]. Indeed, the boronate inhibitor PS-341 (developed originally by ProScript and now in clinical development by Millennium Pharmaceuticals), after showing impressive anti-proliferative effects in several animal model systems and cell culture [79,132,133], is currently in NCI-sponsored human clinical trials for the treatment of many different forms of cancer [134]. The results of phase I trials showed surprisingly low toxicity and no bone marrow suppression at concentrations that appear to cause significant inhibition of the proteasome (J. Adams, Millennium Pharmaceuticals, personal communication).

6.5. *Adaptation and resistance to proteasome inhibitors*

Although prolonged exposure to proteasome inhibitors is toxic to almost all cells, in one cell line (EL-4), a small fraction of cells (0.3%) exposed to the proteasome inhibitors NLVS and β-lactone were able to escape death and to multiply after a period of adaptation [86,135]. It was originally concluded that proteasome function was completely lost in these adapted cells, and that an alternative pathway for proteolysis existed [135]. However, subsequent analysis demonstrated that, while chymotrypsin-like activity is indeed completely inhibited, two other active sites retain some activity [89]. The exact mechanism of adaptation is unclear, but tripeptidyl peptidase II (TPPII), a very large cytosolic oligopeptidase, is induced in adapted cells, and overexpression of this peptidase has been found to increase the cells' resistance to NLVS [86,89]. Presumably, increased levels of TPPII and possibly other peptidases can compensate for the impaired proteasomal activity, which would be insufficient for survival under normal circumstances, perhaps by helping the cell to remove partially digested fragments.

It has recently been reported that Burkitt's lymphoma cell lines are resistant to apoptosis induced by proteasome inhibitors [136]. Although proteasomal peptidase activities were significantly reduced in these cells, the overall rates of protein breakdown were not changed. As in NLVS-adapted cells, in Burkitt lymphoma cells it was found that the activity and content of TPPII are increased, and this effect appears to be due to the constitutively activated c-*myc* oncogene. Moreover, the inhibitor of TPPII Ala-Ala-Phe-chloromethylketone (AAF-cmk), in contrast to proteasome inhibitors, was able to inhibit proliferation of these cells, suggesting that upregulation of TPPII can indeed compensate for the decreased overall activity of proteasome in these cancer cell lines. However, this chloromethylketone also inhibits aminopeptidases [137] and may alkylate cellular proteins.

6.6. *Anti-inflammatory activity of proteasome inhibitors*

The critical biochemical event in the initiation of the inflammatory response is the rapid destruction of the inhibitory protein IκB which occurs in response to various toxic stimuli. IκB was the first substrate of the ubiquitin–proteasome pathway identified with the help of proteasome inhibitors [75]. IκB is an inhibitor of transcription factor NF-κB, which activates the expression of many genes encoding inflammatory mediators (e.g. tumor necrosis factor, IL-1), enzymes (cyclooxygenase, nitric oxide synthetase) and leukocyte adhesion molecules (ICAM, VCAM) [138]. Consequently, in cultured cells and in

vivo, proteasome inhibitors, by stabilizing IκB, maintain NF-κB in the inhibited state and prevent production of these proteins. In animal models the proteasome inhibitors PS-341 and epoxomicin (Fig. 5) exhibit potent effects against arthritis and other inflammatory conditions [12,13,139]. The potent analogue of lactacystin-β-lactone PS-519 (Fig. 5) [140] has anti-inflammatory effects in animal models of asthma [141]. Moreover, this compound was shown to reduce reperfusion injury in the brain [142] and improve neurological recovery after ischemic injury [143], and it is currently in phase I clinical trials for the treatment of stroke. On the other hand, proteasome inhibition stabilizes short-lived cyclooxygenase II, a critical enzyme involved in synthesis of inflammatory prostaglandins in the neuronal cell line, which by itself can be potentially pro-inflammatory [119].

6.7. Anti-HIV effects of proteasome inhibitors

Recently several groups of investigators demonstrated that proteasome inhibitors block release and maturation of HIV and other retroviruses (reviewed in [144]). The effect appears to be indirect, due to the depletion of pool of free ubiquitin, whose conjugation to viral Gag polyproteins is required for virus release from the cell but does not target Gag proteins for proteasomal degradation. Nevertheless, it has even been suggested that proteasome inhibitors can be used for the treatment of HIV infection [145].

6.8. Inhibition of antigen presentation

The proteasome also plays a key role in immune surveillance against viruses and cancer [14]. Protein degradation within the cells is the source of the 8–10 residue antigenic peptides presented to the cytotoxic T-lymphocytes on the surface of the cells in association with major histocompatibility (MHC) class I molecules. The first in vivo study of proteasome inhibitors demonstrated that blocking the proteasome reduces the generation of peptides used in MHC class I antigen presentation [5]. Apparently, a fraction of peptides generated during continuous degradation of cell proteins escape complete destruction to amino acids, are taken up by the endoplasmic reticulum and then transported to the cell surface bound to MHC molecules. Subsequent studies demonstrated that proteasome is absolutely essential for the generation of the C-terminus of the MHC class I ligands, while their N-termini can be generated as a result of trimming of proteasomal products by cytosolic aminopeptidases (reviewed in [14]).

Interestingly, while presentation of the majority of epitopes is reduced by proteasome inhibitors, presentation of a few others was stimulated by low concentrations of these compounds, leading some investigators to conclude that other proteinases may be involved in the generation of these epitopes (reviewed in [14]). However, at higher concentrations, which block all proteasome function, presentation of these antigens is inhibited, and the alternative explanation of these data would be that the chymotrypsin-like site, the primary target of inhibitors used in those experiments, both generates and destroys these epitopes [146]. Now that cell-permeable and site-selective inhibitors of caspase-like sites are available (see below) and when such compounds become available for the trypsin-like site, it should be possible to determine whether these two sites also generate or destroy epitopes in a similar fashion. Indeed, recent in vitro experiments with purified 26S proteasomes demonstrated that epitope is generated only a small fraction of the time that a protein is degraded, and, most often, proteasomes cut within the epitope [147].

This role of the proteasome in both defending against viruses and cancer is also regulated by cytokines, especially by the immune activator IFN-γ. In fact, exposure of cells to IFN-γ causes the induction of three alternative catalytic subunits, LMP2 (β1i), LMP7 (β5i) and MECL1 (β2i), which enhance the production of MHC class I ligands or their precursors [14].

7. Subunit-specific inhibitors

As discussed above, specific inhibitors of individual active sites could be valuable tools to study antigen presentation. As all the active sites cleave peptide bonds by the same catalytic mechanism, subunit specificity of the inhibitors cannot be achieved by using different pharmacophores, and one needs to define in detail differences in the substrate binding pockets of three active sites. The majority of proteasome inhibitors currently available primarily inhibit the chymotrypsin-like activity, but none of them are highly specific, because they also partially block at least one of the remaining two activities (Table 3). However, systematic replacement of residues in the peptide portion of epoxomicin led to the generation of much more selective Ac-hPhe-Leu-Phe-Leu-EX (YU101), which was not only four-fold more potent against chymotrypsin-like activity [148], but inactivated two other activities at 8–24 000-fold slower rates (Table 3).

The specific inhibitor of trypsin-like activity, the peptide aldehyde maleinimido-βAla-Val-Arg-al, is a good example of structure-based design [149]. The maleinimide moiety of this inhibitor reacts covalently with the free thiol group of the cysteine residue in the S3 binding pocket of the trypsin-like site, resulting in its selective and irreversible inhibition. However, this compound is very likely to be inactivated within cells as a result of the reaction of maleinimide with glutathione, limiting the application of this inhibitor only to cell-free systems.

Recently, Nazif and Bogyo [90] made the interesting observation that several peptide vinyl sulfones with an asparagine residue in the P1 position reacted with β2 sub-

units, responsible for the trypsin-like activity, faster than with other subunits. Using positional scanning combinatorial libraries, these authors generated potent and highly selective inhibitors of the trypsin-like activity, Ac-Pro-Arg-Leu-Asn-VS and Ac-Tyr-Arg-Leu-Asn-VS. Unfortunately, these compounds appear not to enter cells readily (M. Bogyo, personal communication).

AEBSF (Pefablock) is another compound that irreversibly inactivates the trypsin-like site without affecting two others [54]. This general inhibitor of serine proteinases has been used in vitro to study the role of the trypsin-like site in protein breakdown by purified proteasomes [46].

Specific inhibitors of the caspase-like site were also generated recently. A cell-permeable epoxyketone, Ac-Gly-Pro-Phe-Leu-EX (YU102), inactivates the caspase-like activity 50-fold faster than chymotrypsin-like activity [57]. The peptide aldehyde Ac-Ala-Pro-Nle-Asp-al is even more selective, and has a K_i for the caspase-like activity below 1 μM, but its cell permeability has not been tested yet (A. Kisselev et al., in preparation). The physiological consequences of inhibition of this site are unclear.

8. Conclusions

The last few years have witnessed major progress in our understanding of intracellular protein degradation. These advances have in part been made possible by the discovery of synthetic and naturally occurring inhibitors of proteasomal proteolytic activities. We expect that subunit-specific, cell-permeable inhibitors will be added to this palette of reagents in coming years. Of particular interest will be the generation of inhibitors of non-proteolytic components of the 26S proteasome and of the ubiquitin conjugation enzymes. Recent developments in combinatorial chemistry and high-throughput screening and structure-based design, as well as new assays of in vivo protein breakdown [150,151], should speed up identification of such molecules and may also lead to the new non-peptide inhibitors of the proteasome. Use of genomics and proteomics approaches will allow the analysis of the effects of these inhibitors on global patterns of gene expression and protein composition of the cell, paving the way to a much better understanding of the biological roles of the proteasome.

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